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Research Article

Inhibition of xanthine oxidase by liquiritigenin and isoliquiritigenin isolated from *Sinofranchetia chinensis*

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Abstract. The methanol extract of the stem of *Sinofranchetia chinensis* inhibited the activity of xanthine oxidase in vitro. Bioassay-guided purification led to the isolation of liquiritigenin and isoliquiritigenin as the main xanthine oxidase inhibitors. This inhibition of enzyme activity was found to be dose dependent, with an IC_{50} value of approximately 49.3 μM for liquiritigenin and 55.8 μM for isoliquiritigenin. Lineweaver-Burk transformation of the inhibition data indicated that the inhibition was of a mixed type for both liquiritigenin and isoliquiritigenin. For liquiritigenin, the K_i and K_I were determined to be 14.0 μM and 151.6 μM, respectively. For isoliquiritigenin, the K_i and K_I were determined to be 17.4 μM and 81.9 μM, respectively. These results suggest that these natural products could be used to treat conditions where the inhibition of xanthine oxidase is warranted.

Key words. *Sinofranchetia chinensis*; Lardizabalaceae; liquiritigenin; isoliquiritigenin; xanthine oxidase.

Gout is a common disease which affects a substantial proportion of the adult population. Hyperuricemia leads to the accumulation of uric acid in joints and kidneys causing acute arthritis and uric acid nephrolithiasis. One therapeutic approach for gout is the use of xanthine oxidase inhibitors such as allopurinol [1, 2], which blocks the synthesis of uric acid from purines. However, allopurinol use can result in a number of adverse side effects [3], ranging from mild skin allergy to a concerted allopurinol hypersensitivity syndrome which can sometimes be life threatening [4–7]. Thus there is a need to develop compounds with xanthine oxidase inhibitory activities but devoid of the undesirable effects of allopurinol. A potential source of such compounds is medicinal materials of plant origin which are used to treat conditions akin to gouty arthritis.

Inhibition of xanthine oxidase is also an important strategy in the formulation of preservation media for organ transplantation purposes. To preserve the organ during transit, ischemia-reperfusion damage must be minimized. Such damage is mediated, in part at least, by xanthine oxidase-derived superoxide anion radicals [8]. These oxyradicals have been associated with postischemic tissue injury, edema, and changes in vascular permeability [9, 10]. Thus, xanthine oxidase inhibitors of one kind or another are found in various organ preservation formulae [11, 12]. In addition, inhibition of xanthine oxidase has been suggested for the treatment of hepatitis and brain tumor because increased serum

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xanthine oxidase levels are found in hepatitis and hepatotoxicity [13] as well as in brain tumors [14].

The stem of *Sinofranchetia chinensis* (D.C.), belonging to the family of Lardizabalaceae and distributed in the western part of China (Yunnan, Sichuan, Hubei, Gansu, Shanxi), has been used in China as a traditional herb to treat dysuria and painful joints of the limbs. Preliminary results have shown that the methanol extract of this herb exhibits an inhibitory action on xanthine oxidase. The enzyme inhibition assay leads to the isolation of liquiritigenin and isoliquiritigenin (fig. 1) as the major xanthine oxidase inhibitors in this species. The present paper reports the characterization of these enzyme-inhibitory actions.

Materials and methods

Reagents. Xanthine was purchased from Sigma (USA). Xanthine oxidase, of bovine milk origin, was purchased from Boehringer Mannheim (Germany). All other reagents used were of analytical grade.

Plant material. The air-dried stems of *S. chinensis* were collected in Gannan Perfecture, Gansu Province, China. A voucher specimen, identified by L. X. Zhang, was deposited in the Herbarium of Nanjing University, Nanjing, China.

Isolation and structure identification of liquiritigenin and isoliquiritigenin. Briefly, the air-dried plant (910 g) was pulverized and extracted twice with methanol at room temperature. The extract was concentrated under reduced pressure and chromatographed on a silica gel column eluted with a chloroform/methanol gradient to give five fractions. Xanthine oxidase-inhibitory activity was found in the fourth fraction (the IC_{50} is equivalent to about 13.4 g of dried plant material per milliliter). This fraction was further fractionated successively over a silica gel column (eluted with a chloroform/methanol gradient) and a Sephadex LH-20 column (eluted with a 1:2 chloroform/methanol mixture). Bioassay-guided purification led to the isolation of liquiritigenin (30 mg) and isoliquiritigenin (61 mg). Structure elucidation was performed by a combination of spectral methods [infrared (IR), mass spectrometry (MS), ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR)]. All NMR experiments were done on a Joel JNM-A 500 FT-NMR spectrometer using tetramethylsilane as the internal standard. Mass spectra were recorded on a ZAB-HS mass spectrometer. IR spectra were taken on a Nicolet 170SX FT-IR spectrometer. The structures of liquiritigenin and isoliquiritigenin are shown in figure 1.

Xanthine oxidase assay. Xanthine oxidase (EC 1.1.3.22) activity was assayed by reacting the enzyme with xanthine under aerobic conditions [15]. Unless otherwise stated in experiments where changes were necessary, the standard assay procedure for xanthine oxidase is as follows. The assay was performed in a final volume of 1 ml 100 mM sodium pyrophosphate buffer pH 7.5 in a quartz cuvette at 25 °C. After equilibration, 0.1 units of the enzyme and 100 µl of 1.2 mM xanthine were then added. Xanthine was dissolved in the assay buffer with gentle heating and was shaken well until completely dissolved before use. The absorbance increase at 295 nm representing the formation of uric acid was followed spectrophotometrically and the initial velocity calculated. The isolated compounds, dissolved initially in dimethyl sulfoxide (DMSO), were incorporated in the enzyme assay to assess their inhibitory activity at different concentrations, in comparison with allopurinol as the standard inhibitor. The final concentration of DMSO in the assay was 5%. Proper controls with DMSO were carried out. DMSO, at a final concentration of 5%, did not affect the enzyme assay.

The enzyme exhibited Michaelis-Menton-type kinetics with a K_m value of 30 µM. Using a molar absorptivity value of uric acid of 1.22×10^4 cm^{-1} [16], V_{max} was calculated to be 0.11 µmol uric acid per minute at 25 °C. In the standard assay, a substrate concentration of 120 µM was employed. Under such assay conditions,

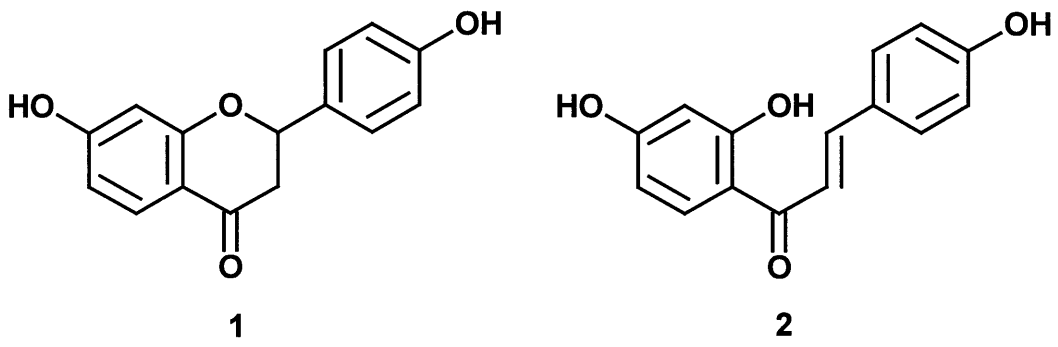

Figure 1. Structure of liquiritigenin (1) and isoliquiritigenin (2).

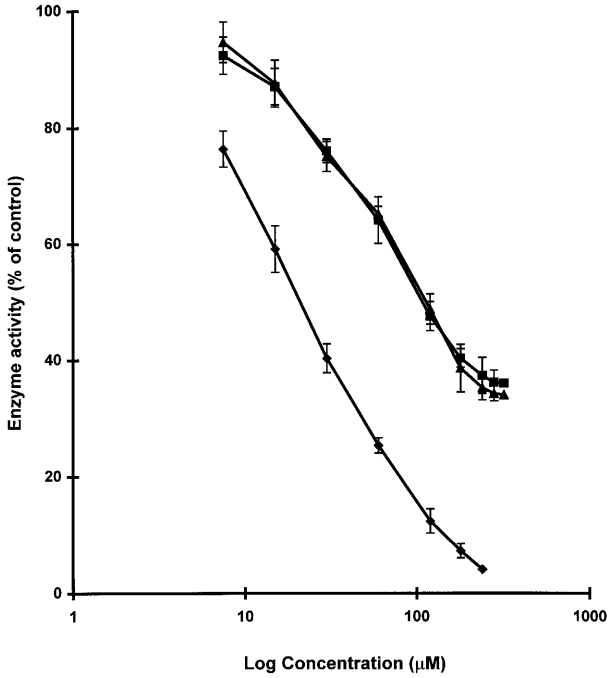

Figure 2. Dose-dependent inhibitory actions of liquiritigenin, isoliquiritigenin, and allopurinol on xanthine oxidase. Xanthine oxidase assays were performed as described in Materials and methods. Different concentrations of liquiritigenin (■), isoliquiritigenin (▲), and allopurinol (◆) were incorporated in the assays. Results are expressed as percentage of control where no inhibitor was added. Data are the average of four independent experiments and error bars indicate standard deviations.

the enzymatic reaction was linear up to at least 1 min, approaching a plateau thereafter, possibly due to substrate depletion. In the standard assay, the initial velocity was always determined within the first 10–20 s. Working in the initial linear portion of the progression curve is particularly important for assays performed at low substrate concentrations in the subsequent kinetics work.

Results

Structure confirmation of liquiritigenin and isoliquiritigenin. Bioassay-guided purification led to the isolation of liquiritigenin and isoliquiritigenin as the main xanthine oxidase inhibitors from the methanol extract of the stems of *S. chinensis*. Liquiritigenin and isoliquiritigenin are known compounds which have been isolated and identified from plants [17, 18]. The confirming spectroscopic data for liquiritigenin are as follows. ^{1}H-NMR (500 MHz, DMSO-d_6): δ 5.45 (1H, dd, J = 13.2, 2.3 Hz, H-2), 3.05 (1H, dd, J = 16.9, 13.3 Hz, H-3α), 2.67 (1H, dd, J = 16.9, 3.2 Hz, H-3β), 7.73 (1H, d, J = 8.2 Hz, H-5), 6.58 (1H, dd, J = 8.2, 2.1 Hz, H-6), 6.42 (1H, d, J = 2.1 Hz, H-8), 7.41 (2H, d, J = 8.3 Hz, H-2′ and 6′), 6.90 (2H, d, J = 8.3 Hz, H-3′ and 5′), 8.90 (2H, br s, HO-4′ and HO-7). ^{13}C-NMR (125 MHz, DMSO-d_6): δ 192.7 (C, C = 0), 81.3 (CH, C-2), 45.1 (CH_2, C-3), 129.1 (C, C-5), 110.3 (C, C-6), 165.4 (C, C-7), 104.7 (C, C-8), 115.9 (C, C-4α), 166.1 (C, C-8α), 130.9 (C, C-1′), 129.1 (C, C-2′), 116.4 (C, C-3′), 159.0 (C, C-4′), 117.0 (C, C-5′), 128.6 (C, C-6′). The confirming spectroscopic data for isoliquiritigenin are as follows. ^{1}H-NMR (500 MHz, DMSO-d_6): δ7.79 (1H, d, J = 15.5 Hz, H-α), 7.86 (1H, br d, J = 15.5 Hz, H-β), 7.76 (2H, br d, J = 8.4 Hz, H-2 and H-6), 6.95 (2H, d, J = 8.4 Hz, H-3 and H-5), 6.38 (1H, d, J = 2.1 Hz, H-3′), 6.48 (1H, dd, J = 8.2, 2.1 Hz, H-5′), 8.15 (1H, d, J = 8.2 Hz, H-6′), 13.66 (1H, s, HO-2′), 9.15 (2H, br s, HO-4 and HO-4′). ^{13}C-NMR (125 MHz, DMSO-d_6): δ192.5 (C, C = O), 118.2 (CH, C-α), 145.1 (CH, C-β), 127.1 (C, C-1), 131.7 (CH, C-2 and C-6), 116.7 (CH, C-3 and C-5), 161.0 (C, C-4), 116.8 (C, C-1′), 167.6 (C, C-2′), 103.7 (CH, C-3′), 165.6 (C, C-4′), 108.7 (CH, C-5′), 133.3 (CH, C-6′).

Liquiritigenin and isoliquiritigenin inhibition of xanthine oxidase. Micromolar concentrations of liquiritigenin or isoliquiritigenin, when incorporated into the xanthine oxidase assay, elicited dose-dependent inhibition of enzyme activity (fig. 2). The dose-dependent curves of liquiritigenin and isoliquiritigenin appear to be superimposable, and they parallel the allopurinol dose-dependent curve. Apart from the fact that allopurinol is more potent than liquiritigenin and isoliquiritigenin, there is another observable difference between the xanthine oxidase inhibitory actions of allopurinol and the natural compounds. At sufficiently high concentrations of allopurinol, enzyme inhibition was complete. However, about 35% of the enzyme activity remained uninhibited at elevated concentrations of liquiritigenin and isoliquiritigenin. The reason for this residual activity is at present not known. The concentration of these natural compounds at which 50% of the inhibitable xanthine oxidase activity was inhibited (IC_{50}) was estimated to be 49.3 μM for liquiritigenin and 55.8 μM for isoliquiritigenin. The IC_{50} of allopurinol was determined to be 24.4 μM, similar in magnitude to values previously reported by a number of investigators [19–23].

For xanthine oxidase assays performed as a function of different concentrations of xanthine in the absence or presence of liquiritigenin, Lineweaver-Burk transformation of the data is shown in figure 3. Parallel studies were carried out with isoliquiritigenin, as shown in figure 4. The data indicate that the mode of xanthine oxidase inhibition by both liquiritigenin and isoliquiritigenin is of the mixed type. In our hands, allopurinol also acts as a mixed inhibitor of xanthine oxidase, as reported by other investigators [24, 25].

In mixed inhibition, the inhibitor can bind to the free enzyme as well as to the enzyme-substrate complex [26].

As a result, two inhibitor constants, K_i and K_I, can be defined, where K_i is the dissociation constant of the enzyme-inhibitor complex, and K_I is the dissociation constant of the enzyme-substrate-inhibitor complex.

Since the Lineweaver-Burk plots of liquiritigenin (fig. 3), isoliquiritigenin (fig. 4), and allopurinol (not shown) cross to the left of the 1/V axis but above the 1/[S] axis, they all belong to the so-called competitive-noncompetitive type of inhibition where $K_I > K_i$.

K_i can be calculated from the slope of the inhibited curve where:

$$\text{Slope} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)$$

K_I can be calculated from the y-intercept of the inhibited curve where:

$$\text{y-intercept} = \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_I}\right)$$

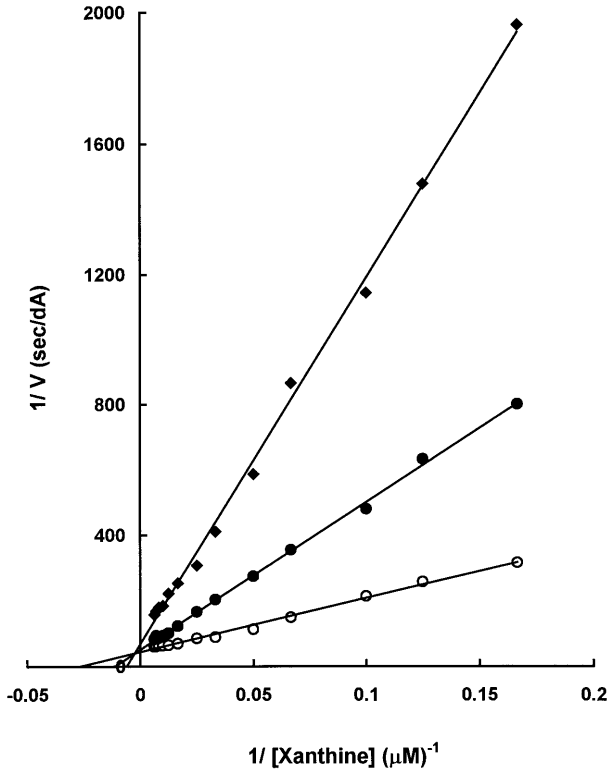

Figure 3. Lineweaver-Burk plot of xanthine oxidase inhibition by liquiritigenin. Xanthine oxidase assays were performed as described in Materials and methods, but with various concentrations of xanthine. Each experiment comprised three conditions: the control without any inhibitor (○), in the presence of 54.7 μM liquiritigenin (●), and in the presence of 115.2 μM liquiritigenin (◆). Enzyme activity was expressed as change in absorbance at 295 nm per unit time. The Lineweaver-Burk-transformed data were plotted, followed by linear regression of the points. Data represent the average of two experiments.

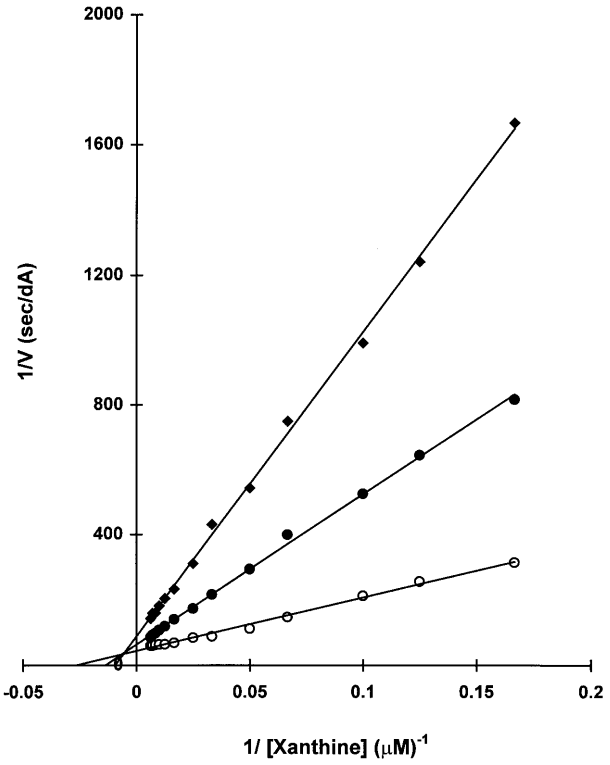

Figure 4. Lineweaver-Burk plot of xanthine oxidase inhibition by isoliquiritigenin. The experimental conditions and the subsequent data analyses were the same as in Figure 3, except that isoliquiritigenin was used instead of liquiritigenin: control without any inhibitor (○), in the presence of 40 μM isoliquiritigenin (●), and in the presence of 118.8 μM isoliquiritigenin (◆). Data represent the average of two experiments.

A comparison of the inhibitory characteristics of the three compounds is summarized in table 1.

Discussion

Recent findings show that the occurrence of gout is increasing in China [27], possibly due to changes in dietary habits. The treatment of gout entails the use of several classes of therapeutic agents such as xanthine oxidase inhibitors. Among the xanthine oxidase inhibitors used, allopurinol is the most widely employed. However, adverse side effects associated with the use of allopurinol are not uncommon and a search for substitutes is therefore highly warranted. We thus began our program to look for xanthine oxidase inhibitors of phytochemical origin from Chinese medicinal materials. The crude methanol extract of *S. chinensis* exhibited xanthine oxidase inhibitory activity in vitro and the active principles were identified as liquiritigenin and isoliquiritigenin. These experimental findings corroborate the customary use of *S. chinensis* in China as a

Table 1. Inhibition of xanthine oxidase by allopurinol, liquiritigenin, and isoliquiritigenin: a comparison.

Inhibitor	IC_{50} (μM)	Type of inhibition	K_i (μM)	K_I (μM)
Allopurinol	24.4	mixed	7.3	28.9
Liquiritigenin	49.3	mixed	14.0	151.6
Isoliquiritigenin	55.8	mixed	17.4	81.9

herbal medicine to treat painful joints of the limbs. Although both liquiritigenin and isoliquiritigenin were less potent than allopurinol in vitro (table 1), that they might be safer confers a great advantage on these natural compounds. It is worth noting that no overt toxicity has been reported during the use of *S. chinensis* in China. Obviously, the full toxicological profile of liquiritigenin and isoliquiritigenin and their in vivo hypouricemic effects have to be established before their therapeutic potentials can be substantiated. However, these compounds constitute plausible lead candidates for further investigations.

As mentioned, the presence of liquiritigenin and isoliquiritigenin is not limited to *S. chinensis* [17, 18]. Indeed, licorice has been reported to be another rich source of liquiritigenin and isoliquiritigenin [28–30], and isoliquiritigenin has also been isolated from soybean [31]. This is an important point, because other plant materials could act as alternative sources of these potentially useful compounds.

Drug interaction is another consideration. Allopurinol is known to interact with a number of drugs [32]. For example, allopurinol would enhance the bone marrow toxicity of 6-mercaptopurine in cancer patients [33, 34]. Other examples of drug interaction include cyclophosphamide, theophylline, azathioprine, phenytoin, and thiazide diuretics. The availability of a completely different class of compounds as inhibitors might circumvent such problems. Further studies are warranted.

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